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METHOD FOR INHIBITION OF VIRAL MORPHOGENESIS

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Technical Field

The invention is directed to inhibiting viral morphogenesis and viral infection. In particular, it concerns effecting such inhibition by inhibiting the prenylation or post prenylation reactions of a viral protein.

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Background Art

It has been shown that certain membrane-associated proteins require the addition of lipophilic residues in order to function properly. One family of such modifications is termed "prenylation" because the hydrophobic residue is derived from isoprenoid precursors. The prenyl residue is known to attach to the sulfhydryl group of a cysteine which has been shown in a number of membrane-associated proteins to be contained in a "CXXX" box at the carboxy terminus of the substrate protein. In particular, one such membrane-associated protein has been shown to be the protein product of the ras oncogene. Summaries of these reactions conferring hydrophobic properties on membrane proteins, including prenylation, have appeared by Hoffman, M., Science (1991) 254:650-651, and by Gibbs, J.B., Cell (1991) 65:1-4.

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In addition, in many cases, prenylation is a first step in a series of further reactions which modify the carboxy terminus of prenylated proteins. These prenylation initiated, or post-prenylation reactions include carboxymethylation and proteolysis.

A In the prenylation substrate proteins studied to date, the CXXX box contains aliphatic residues in the second and third positions and a leucine, serine, methionine, cysteine or alanine in the terminal position. Thus, in the CXXX boxes so far studied, the box itself is relatively hydrophobic.

A 15 It has now been found that prenylation of a viral protein is necessary for the morphogenesis of hepatitis delta virus (HDV). This is the first demonstration that viral proteins are subject to prenylation. Furthermore, certain functional consequences can be ascribed to prenylation. The viral protein which is the target of prenylation, surprisingly, contains a hydrophilic CXXX box of the sequence Cys-Arg-Pro-Gln. Prenylation, or prenylation-initiated modification, of this relatively hydrophilic CXXX box and corresponding CXXX boxes (hydrophilic or otherwise) or other cysteine-containing sequences near the C-terminus of proteins in other virions are suitable targets for antiviral strategies.

A 20 These targets can now be seen to include, but are not limited to, proteins of hepatitis A virus (HAV), hepatitis C virus (HCV), herpes simplex virus (HSV), cytomegalovirus (CMV), varicella-zoster virus (VZV), influenza virus, plant viruses such as tobacco mosaic satellite virus (TMSV) and barley stripe mosaic virus (BSMV), the core antigen of hepatitis B virus (HBV) and the nef gene product of human immunodeficiency virus-1 (HIV-1) -- especially since nef has been shown to play an

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important role in the development of AIDS. (Kesstler, H.W. III, et al. Cell (1991) 65:651-662. Accordingly, inhibition of the prenylation of these target proteins or the post-prenylation reactions thereof is claimed to be inhibitory to the progress of these infections.

Disclosure of the Invention

The invention provides methods to interfere with viral morphogenesis, production, release or uncoating both *in vitro* and *in vivo*. Agents which interfere with the prenylation of, or the post-prenylation reactions of, at least one viral protein are provided to infected cells to halt the viral infection. Such cells may be in culture or may be contained in an animal or plant subject.

Thus, in one aspect, the invention is directed to a method to inhibit viral morphogenesis, production, release or uncoating which method comprises effectively interfering with the prenylation of, or the post-prenylation reactions of, at least one viral protein. In another aspect, the invention is directed to an assay method for screening candidate drugs for their ability to inhibit prenylation. In a third aspect, the invention is directed to a method for treating viral infection by administering an agent effective to inhibit prenylation of, or the post-prenylation reactions of, a viral protein. In preferred embodiments, the viral protein is the large delta antigen of the hepatitis D virus, core antigen of HBV, or the nef protein of HIV.

Brief Description of the Drawings

Figures 1A and 1B are photocopies of immunoblots of proteins obtained by lysis of viral-

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infected cells expressing viral proteins and treated with tritiated mevalonate.

Figures 2A and 2B are photocopies of immunoblots of proteins derived from lysates of cells containing wild type or mutant viral proteins and labeled with tritiated proline or mevalonate.

Figures 3A, 3B, 3C and 3D are photocopies of immunoblots of various cell supernatants containing viral proteins.

Figure 4 is a diagrammatic representation of the progress of HDV morphogenesis.

Modes of Carrying Out the Invention

Hepatitis delta virus (HDV) infections cause both acute and chronic liver disease and can be fatal (1, 2). This RNA virus contains a 1.7 kb single-stranded circular genome and delta antigen, the only known HDV-encoded protein. These elements are encapsulated by a lipid envelope in which hepatitis B virus surface antigens are embedded (3), which explains why HDV infections occur only in the presence of an accompanying HBV infection (4, 5). Two isoforms of delta antigen exist in infected livers and serum (6, 7). This heterogeneity arises from a unidirectional mutation at a single nucleotide in the termination codon for delta antigen (codon 196: UAG → UGG), which occurs during replication (8). Thus, although small delta antigen is 195 amino acids long, large delta antigen is identical in sequence except that it contains an additional 19 amino acids at its COOH terminus. Although both forms of delta antigen contain the same RNA genome binding domain (9), they have dramatically different effects on genome replication. The small form is required for replication,

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whereas the large form is a potent trans-dominant inhibitor (10, 11).

A 5 The last four amino acids of large delta antigen are Cys-Arg-Pro-Gln-COOH. (SEQ ID NO: 2) This COOH-terminal configuration, termed a CXXX box (SEQ ID NO: 1) (where C is cysteine and X is any amino acid), has been implicated as a substrate for prenyltransferases that add to the cysteine 15 (farnesyl) or 20 (geranylgeranyl) carbon moieties derived from mevalonic acid (12-14). The resulting hydrophobic
10 modification may aid in membrane association of the derivatized protein, as suggested for p21 Ras (15, 16) and lamin B (12, 17). We have now demonstrated that large delta antigen is similarly modified.

A 15 Other virions also contain suitable target sequences for prenylation. These sequences are near the carboxy terminus of the viral protein targeted, and may be in the form of CXXX boxes, (SEQ ID NO: 1) but the cysteine may also be closer to the C-terminus, including a position as the C-terminal amino acid, as is the case of the core antigen
20 of hepatitis B virus (HBV) and the nef gene product of HIV-1.

To determine whether large delta antigen is a substrate for prenylation, we labeled three cell lines, SAG, LAG, and GP4F, with [³H]mevalonic acid. GP4F cells
25 are a derivative of NIH 3T3 cells (18). SAG (19) and LAG (20) cells are derivatives of GP4F cells that stably express the small and large delta antigens, respectively.

Labeled cell lysates were analyzed on immunoblots (Fig. 1A) to detect steady-state amounts of
30 small and large delta antigen. The lysates were also subjected to immunoprecipitation with an antibody to the delta antigens (anti-delta), SDS polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography (Fig. 1B).

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In more detail, referring to Fig. 1, large delta antigen is shown to be prenylated in cultured cells. The cell lines SAG (19) (lane 1), LAG (20) (lane 2), and GP4F (18) (lane 3) were grown overnight in Lovastatin (25 μ M) and (R,S)-[5-³H]mevalonate (140 mM) (30), and lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] (20). (A) Aliquots were subjected to immunoblot analysis (11). The blot was treated with serum from an HDV-infected patient that contained antibody to delta antigen (α - δ Ag) and horseradish peroxidase-conjugated rabbit antibody to human immunoglobulin G (IgG) (Promega), followed by chemiluminescence (Amersham) development. (B) Immunoprecipitates (with α - δ Ag) from cell extracts were subjected to SDS-PAGE and fluorography. As shown in Figure 1, S, small delta antigen, L, large delta antigen. Molecular size markers are shown at the left (in kilodaltons).

Thus, the large, but not the small, antigen was labeled with [³H]mevalonic acid, suggesting that large delta antigen undergoes prenylation in cultured cells.

We obtained similar results using *in vitro* translation reactions (13) performed in the presence of [³H]proline or [³H]mevalonate (Fig. 2). Fig. 2. also shows mutation of Cys²¹¹ of large delta antigen to Ser and loss of prenylation. *In vitro* translation reactions were performed with rabbit reticulocyte lysates (Promega) in the presence of either (A) L-[2,3,4,5-³H]proline (19 μ M) (94 Ci/mmol, Amersham) or (B), [³H]mevalonate (200 μ M) (30). For (A) and (B), translation reactions contained small delta antigen mRNA (lane 1); large delta antigen mRNA (lane 2); water (lane 3); or large delta antigen (Cys²¹¹ \rightarrow Ser) (20) mRNA (lane 4). A portion (20 μ l) of each reaction was added to 1 ml of RIPA buffer,

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immunoprecipitated with α - δ Ag, and analyzed as described (Fig. 1).

Both the small and the large antigens were labeled with [3 H]proline (Fig. 2A), whereas only the large isoform was labeled with [3 H]mevalonate (Fig. 2B). To determine whether modification by [3 H]mevalonate was dependent on the presence of Cys²¹¹ in the terminal CXXX₁ box, we constructed a mutant that contains a serine at this position (20). Cys²¹¹ is the only cysteine in large delta antigen. Mutating Cys²¹¹ to Ser did not interfere with the synthesis of large delta antigen (Fig. 2A) but abolished its modification by [3 H]mevalonate (Fig. 2B). (SEQ ID NO: 1)

The specific type of mevalonate modification of large delta antigen appears to be geranylgeranyl rather than farnesyl (21). Although the first described CXXX₁ boxes contained aliphatic residues at the first and second positions after Cys, other types of amino acids can be found in prenylation sites (13, 14). It is not clear whether the COOH-terminal sequence Cys-Arg-Pro-Gln-COOH, which differs from that of previously described CXXX₁ boxes, implies the existence of a novel prenylation enzyme or whether it reflects a broader substrate specificity of known prenyltransferases. (SEQ ID NO: 2) (SEQ ID NO: 1)

For HDV particle formation, delta antigen and associated genomes are presumably targeted to cell membranes that contain HBV envelope proteins. We hypothesized that prenylation of large delta antigen could be involved in this process. We first examined whether large delta antigen was sufficient for HDV-like particle formation. HBV surface antigen (HBsAg) was expressed transiently in COS-7 cells together with small or large delta antigen. Virus-like particles consisting of delta antigen packaged into HBsAg-containing envelopes

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were analyzed by immunoprecipitation of clarified media supernatants with an antibody to HBsAg (anti-HBs).

Fig. 3 shows particle formation with large delta antigen and HBsAg parts. For panels (A) and (B), COS-7 cells were transiently transfected with the following plasmids: SV24H, which expresses HBV surface antigen (31), and SVLAg, which expresses small delta antigen (19) (lane 1); SV24H and SVL-large, which expresses large delta antigen (20) (lane 2); and calcium phosphate precipitate without DNA (lane 3). In (C) and (D), COS-7 cells were transfected with SV24H and SVL-large (lane 4); SV24H and SVL-large (Ser²¹¹) (20) (lane 5); and calcium phosphate precipitate without DNA (lane 6). For (A) and (C), 48 hours after transfection, HBsAg-containing particles were immunoprecipitated from 2-ml aliquots of clarified media supernatants with anti-HBs (31) and subjected to immunoblot (with α - δ Ag) and chemiluminescence analyses as described (Fig. 1). For (B) and (D), the transfected cells were harvested in cell lysis buffer [50 mM Tris (pH 8.8), 2% SDS] with protease inhibitors (20), and aliquots subjected to protein immunoblot and chemiluminescence analyses. Molecular size markers are shown at the left (in kD).

The presence of delta antigen in the immunoprecipitates was assayed by immunoblot analysis (Fig. 3A). Although both small and large antigens were synthesized in the transfected cells (Fig. 3B), only the large isoform was incorporated into secreted HBsAg-containing particles (Fig. 3A). Similar selective packaging has been observed (22).

We then examined the function of mevalonate modification in this particle formation. One explanation for the preferred packaging of large delta antigen is

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(SEQ ID NO:1)

A that the small antigen lacks the CXXX box and therefore cannot undergo modification. The Cys²¹¹ → Ser mutant of large delta antigen should behave like small delta antigen and not be packaged. This was indeed found to be the case. Whereas both wild-type and Ser²¹¹ mutant large antigens were synthesized in transfected cells (Fig. 3D), only the wild-type form was packaged into particles (Fig. 3C). Thus, the mutated form of large delta antigen is not prenylated and cannot form particles with HBsAg.

10 Our results suggest that prenylation of large delta antigen is required for the formation and release of particles containing delta antigen and HBV surface antigens. The requirement of a prenylation site for productive viral infection is further suggested by other mutations of the CXXX box (23) and by the conservation of Cys²¹¹ and a CXXX box motif among all sequenced HDV isolates (24).
(SEQ ID NO:1)
(SEQ ID NO:1)

A 15 The ability of large, but not small, delta antigen to be prenylated and packaged into virus particles further highlights the significance of the mutation-induced heterogeneity at the termination codon of the small delta antigen. During HDV replication, S genomes (encoding the small antigen) mutate to L genomes (encoding the large antigen). At least two effects attributable to this mutation can be distinguished (see Fig. 4). Fig. 4 shows the regulatory switch of S genomes to L genomes. During replication, S genomes encoding the small delta antigen mutate to L genomes, which encode the large delta antigen. This single base mutation has two effects on the COOH-terminus of delta antigen. The first is to change the nature of the COOH-terminal amino acid; Pro (P), which enhances genome replication (20), is replaced by Gln (Q), resulting in inhibition of genome replication. The second effect is the creation of a

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(SEQ ID NO: 2)

A target prenylation site (CRPQ), C, cysteine; R, arginine; P, proline; Q, glutamine.

Thus, the first effect is the conversion of an enhancer of genome replication (small delta antigen) into
5 a potent trans-dominant inhibitor (large delta antigen) (10, 11). This dramatic difference in function appears to be determined solely by the nature of the COOH-terminal amino acid with proline being sufficient to confer enhancer activity (11, 25). The second effect is
10 the addition of a CXXX box to delta antigen, which allows the protein to be prenylated and presumably promotes its incorporation into HBsAg-containing particles. The combined effects of the switch from production of small to large delta antigen thus appear to have two roles: to
15 suppress further genome replication and to promote the onset of packaging and virion morphogenesis.

Our results suggest prenylation as a new target for anti-HDV therapy and for antiviral therapy with respect to other viruses with prenylated proteins. Such
20 therapy is directed at inhibiting virion morphogenesis, production, release and uncoating (functionally the reverse reaction of virion morphogenesis). In light of the increasingly apparent degeneracy of the four C-terminal amino acids required to function as a
25 prenylation substrate, a cysteine located at any of these C-terminal positions is also considered to identify a potential target of antiprenylation therapy.

Several strategies designed to interfere with the prenylation stage of the HDV life cycle may be
30 considered, including drugs that inhibit enzymes along the prenylation pathway, and CXXX box analogs. Both
A therapies have been considered for the inhibition of ras-mediated oncogenic transformation (26). Tetrapeptides
A that correspond to the CXXX box of p21 Ha-Ras inhibit
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prenylation of p21 Ha-Ras *in vitro* (27). Finally, the dual function of large delta antigen in the HDV life cycle suggests a further refinement of a proposed (11) defective interfering particle- (DIP) (28) like therapy aimed at cells infected with actively replicating S genomes. Because L genomes require a source of small delta antigen for replication (19, 29) but, once replicated, produce a potent trans-dominant inhibitor of further replication, a therapeutically administered L genome DIP could be specific for infected cells, as well as possess an inherent shut-off mechanism (11). If the L genome also contained the Cys²¹¹ to Ser mutation, it could encode a delta antigen that not only inhibits replication but also affects packaging.

Accordingly, new approaches to antiviral therapy and inhibition of viral morphogenesis focus on inhibition of the prenylation of, or post-prenylation reactions of, at least one viral protein. This may be effected by contacting cells infected with the target virus with an effective amount of an agent which inhibits the prenylation of, or post-prenylation reactions of, at least one viral protein. Such agents include inhibitors of formation of the prenyl groups which are derivative of the mevalonate synthesis pathway. Other agents include decoys for the target sequence for prenylation, including small peptides, including tetrapeptides and other compounds which mimic the surroundings of the cysteine residue to be prenylated. For example, Reiss, Y., et al. Cell (1990) 62:81-88 report prenylation inhibition by C-A-A-X tetrapeptides. As set forth above, the cysteine residue to be prenylated is generally found at the carboxy terminus of the target protein; although the most common target sequence involves a CXXX box, cysteines positioned closer to the C-terminus may also be targeted;

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X thus, the relevant peptides may include those of the form
(SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6)
XCXX, XXCX, and XXXC. Other agents include derivatives
and mimics of prenyl groups themselves. Other suitable
agents include inhibitors of the prenyltransferase
5 enzymes and of enzymes that catalyze post-prenylation
reactions.

Assay of Candidate Inhibitors

10 The present invention also provides a method to
screen candidate drugs as prenylation inhibitors by
taking advantage of the requirement for prenylation in
order to effect secretion of certain prenylated proteins.
For those proteins for which secretion requires
prenylation, the assay can be conducted in a direct and
15 simple manner. Cells that secrete, or that have been
modified to secrete, a first protein whose secretion is
dependent on prenylation are used as the experimental
cells. A second protein which does not depend on
prenylation for secretion is used as a control. This
20 control protein may be secreted by the same or different
host cells as the first protein. The candidate drug is
applied to cells that secrete both proteins, or to
matched sets of cells that secrete each. Secretion can
readily be assessed by assaying the cell supernatants for
25 the presence or absence of the first and second secreted
proteins using, for example, routine ELISA assays.
Successful candidate drugs will not inhibit the secretion
of the control protein, but will inhibit the secretion of
the protein in the test sample wherein prenylation is
30 required for secretion.

35 The large delta antigen of HDV is a viral
protein for which prenylation is a prerequisite for
secretion. Thus, this protein forms, itself a key part
of a useful test system for the assay. Cells that are

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modified to secrete a protein for which prenylation is not required can be used as controls. If large delta antigen is used as the test protein, it is advantageous to use HBsAg as the control protein in the same cell since HBsAg is also required for secretion of delta antigen.

The foregoing assay, of course, requires that the inhibitor interfere with the prenylation system for large delta antigen or for any other prenylation-controlled secreted protein used in the assay. A range of prenyl transferases and prenyl groups is known to apply to various proteins for which prenylation inhibitors are required or sought. Some of these proteins are not secreted, whether they are prenylated or not; one such example is the protein product of the ras oncogene.

Nevertheless, the assay system described can be employed to screen for inhibitors of prenylation in these nonsecreted proteins by providing the target "CXXX" box characteristic of the nonsecreted protein in place of the corresponding "CXXX" box of the secreted one. The resulting chimeric protein will exhibit the prenylation characteristics of the imported "CXXX" box characteristic of the nonsecreted protein, but retain the ability of the host secreted protein to be passed to the supernatant for assay. Thus, the range of target proteins for which prenylation inhibitors are sought by use of the assay can be expanded to nonsecreted proteins.

The presence of a control system which provides secreted protein not dependent on prenylation is critical. The presence of this control allows candidate inhibitors which merely are toxic to the cells, or which inhibit secretion in general, to be discarded. Prenylation inhibitors identified by one of the

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variations of the above described assay are expected to find use not only in the inhibition of viruses, but also in other processes or disease states -- including but not limited to cancer -- in which a prenylated protein is found to be involved.

Evidently, prenylation of viral proteins is a prerequisite for additional post-prenylation reactions of the proteins such as proteolysis and carboxymethylation. The essential sequence of steps can be interfered with at the most convenient point for the viral protein in question.

Administration of the Inhibitors

Additional viral proteins subject to prenylation can be obtained by screening amino acid sequence data banks for viral proteins which contain a "CXXX" box at the C-terminus. An illustrative list of such proteins includes, for example, specific proteins of HAV, HCV, HSV, CMV, VZV, influenza virus, plant viruses such as tobacco mosaic satellite virus and barley stripe mosaic virus, core antigen of hepatitis B virus and the nef gene product of HIV I, as set forth above. These candidates for suitable prenylation targets can be validated in a manner similar to that described above by providing labeled mevalonic acid to cells infected with or containing the appropriate viruses or viral gene products, and assessing the prenylation status of the viral proteins obtained using incorporation of label as the criterion. Furthermore, the role of prenylation in the morphogenesis of the respective virions, and its suitability as a target for anti-viral therapy, can also be validated in a manner similar to that described above.

If viral morphogenesis, production, release or uncoating are to be inhibited in culture, suitable host

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cells are used to culture the virus, and the agents used in inhibiting prenylation or post prenylation reactions added to the medium. If the infected cells are contained in an animal subject, such as a mammalian subject or in particular a human or other primate subject, the agent used for the prenylation inhibition is generally introduced as a pharmaceutical formulation. Suitable formulations depending on the nature of the agent chosen may be found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA. The routes of administration include standard such routes, including administration by injection, oral administration, and transmucosal and transdermal administration. The choice of formulation will depend on the route of administration as well as the agent chosen. Suitable mixtures of agents can also be used as active ingredients. For administration to plants, formulations which are capable of conducting the active ingredients into plant cells are used as carriers.

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